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Construction of a Novel Recombinant *Bombyx mori* Nuclear Polyhedrosis Virus Producing the Fluorescent Polyhedra

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We have constructed a novel recombinant *Bombyx mori* nuclear polyhedrosis virus (BmNPV) producing the green fluorescent polyhedra. For the production of the fluorescent polyhedra, partial polyhedrin gene containing KRKK as nuclear localization site from the BmNPV polyhedrin gene and the green fluorescent protein (gfp) gene were introduced under the control of p10 promoter of BmNPV. The recombinant BmNPV was stably produced fluorescent polyhedra in the infected Bm5 cells and the morphology of the fluorescent polyhedra was similar to that of wild-type BmNPV. The fluorescent polyhedra had 32 kDa native polyhedrin and 41 kDa fusion protein. From these data, we have further developed a novel BmNPV p10-based transfer vector producing recombinant polyhedra with foreign gene product. The novel BmNPV p10-based transfer vector is composed of partial polyhedrin gene, factor Xa, and multiple cloning sites.

Key words : Baculovirus, *Bombyx mori* nuclear polyhedrosis virus, Polyhedra, Green fluorescent protein, Insect cells

Introduction

Baculoviruses have been successfully used as highly efficient eucaryotic expression vectors because they possess several characteristics such as high level expression, authentic biological and immunological activity, and post-translational modification (Luckow and Summers, 1988; Maeda, 1989; O'Reilly *et al.*, 1992). The promoters of the genes encoding the polyhedrin and p10 proteins of baculovirus are most frequently employed in the baculovirus expression vector systems to express heterologous gene (King and Possee, 1992; O'Reilly *et al.*, 1992). *Autographa californica* nuclear polyhedrosis virus (AcNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV) have been already developed as vectors by using both promoters (Kang *et al.*, 1997; Maeda *et al.*, 1985; Smith *et al.*, 1983; Woo *et al.*, 1995).

Both promoters of polyhedrin and p10 proteins are strongly activated during the very late stage of infection. NPVs have a unique feature of producing polyhedra composed polyhedrin in the infected cells. The polyhedrin generally makes up almost 50% of the total protein in the infected cells at a very late stage of infection (Granados and Federici, 1986). Polyhedrin forms the polyhedra and protects the numerous viral progeny. Also polyhedrin itself is nonessential for viral replication. Because of the most abundant expression level and accumulation of polyhedrin in the baculovirus-infected cells, many investigator are interested in the genetic engineering of polyhedra for various purposes of diverse interest (Chung *et al.*, 1980; Yamamoto *et al.*, 1981; Je *et al.*, 2000, 2001). One achievement of the genetic engineering of polyhedra is the recombinant fluorescent polyhedra that produce green fluorescence by recombining polyhedrin gene with a green fluorescent protein (*gfp*) gene (Je *et al.*, 200, 2001). The previous reports introduced recombinant AcNPVs which produce the green fluorescent polyhedra. However, genetic engineering of recombinant polyhedra with foreign gene products has not yet been studied in BmNPV.

In this study we have successfully developed a novel recombinant baculovirus BmNPV which produces the green fluorescent polyhedra. We also have described the recombinant BmNPV producing the fluorescent polyhedra by fusion of partial polyhedrin gene and *gfp* gene

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under the control of BmNPV p10 promoter. Furthermore, we have developed a novel BmNPV p10-based transfer vector producing recombinant polyhedra with foreign gene product.

Materials and Methods

Cells and virus

The *Bombyx mori* 5 (Bm5) (Grace, 1962) cells were grown at 25°C in TC-100 medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL) (O'Reilly *et al.*, 1992). Wild-type BmNPV-K1 (Kang *et al.*, 1997; Park *et al.*, 2000) and recombinant BmNPV, vBm10β-gal (Kang *et al.*, 1997), were propagated and titered in Bm5 cells. The titer was expressed as plaque forming units (PFU) per ml (O'Reilly *et al.*, 1992).

Viral genome isolation

Polyhedra and viral DNA were obtained from Bm5 cells by standard methods (O'Reilly *et al.*, 1992). Polyhedra were purified by centrifugation through discontinuous 40 to 65% sucrose gradients. Viral DNA was isolated from purified polyhedra by proteinase K digestion followed by phenol extraction (O'Reilly *et al.*, 1992).

Polymerase chain reaction (PCR)

Viral DNAs were used as templates. The partial polyhedrin gene of BmNPV was amplified from viral DNAs using the primer 5-GTAGACCCCGGGGGTACCATGC CG-3 and 5-GAGCTCTACAATGGGGAAGCTGTC-3, annealing to the 5 translation start region and 3 structural region, respectively (Woo *et al.*, 1995). After 35-cycle amplification (94°C for 1 min; 65°C for 1 min; 72°C for 30 sec), PCR products were ethanol precipitated, centrifuged at 10,000 g for 15 min, and rinsed with 70% ethanol. These DNAs were analyzed by 1% agarose gel electrophoresis. The PCR products for sequencing were cloned into pGem-T vector (Promega). Sequence was determined using ABI PRISM 377 DNA sequencer (Applied Biosystems, USA).

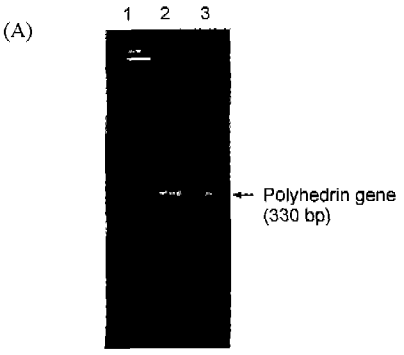
Construction of transfer vector

The amplified 330 bp partial polyhedrin gene of BmNPV was cloned into pGemT vector (Promega) to yield pGTBP 110. The 330 bp partial polyhedrin gene from pGTBP110 was inserted into the *Nco*I and *Kpn*I sites of pEGFP vector (Clontech) to yield pEGFPBP110. The fusion gene with partial polyhedrin and *gfp* genes was eluted from pEGFPBP 110 by digestion of *Bam*HI and *Eco*RI, and then inserted into the *Bam*HI and *Eco*RI sites of pSKII vector to produce pBIISKEGFPBP110. Finally, the fusion gene was eluted from pBIISKEGFPBP110 by digestion of *Eco*RV and *Sma*I, and inserted into the *Sma*I site of pBm10 (Kang *et al.*, 1997) to yield pBm10EGFP-pol transfer vector.

In addition, the partial polyhedrin gene of BmNPV was eluted from pBm10EGFP-pol by PCR and cloned into the *Sal*I and *Sac*I sites of pinpointXa-2 vector (Promega) to yield pXa2-pol110. The partial polyhedrin gene, factor Xa, and multiple cloning sites were eluted from pXa2-pol110 by digestion of *Sma*I and *Bam*HI, and the eluted DNA fragment was inserted into the *Sma*I and *Bam*HI sites of pBm10 vector to produce pBm10pol-Xa. Finally, the pBm10pol-Xa was digested with *Nru*I and *Bam*HI, and *gfp* gene was inserted into the digested sites of pBm10pol-Xa to yield pBm10pol-Xa-GFP.

Construction of recombinant virus

The 35-mm diameter cell culture dish seeded with 1.0 $\times 10^6$ Bm5 cells was incubated at 25°C for 1 hr. One microgram of vBm10β-gal recombinant BmNPV genome (Kang *et al.*, 1997), 5 μg of pBm10EGFP-pol or pBm10 pol-Xa-GFP transfer vector in 20 mM HEPES buffer and sterile water were mixed in a polystyrene tube. Fifty μl of 100 μg/ml Lipofectin (GIBCO/BRL) were gently mixed with 50 μl DNA solution and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC100 medium (GIBCO/BRL). The 1.5 ml serum-free TC100 medium was added



(B)

1 MPNYSYTPTI GRTYVYDNKY YKNLGCLIKN AKRKKHLVEH EQEEKQWDLL DNYMVAEDPF
61 LGPGKNQKLT LFKEIRSVKP DTMKLIVNWS GKEFLRETWT RFVEDSFPIV

Fig. 1. PCR of the partial polyhedrin gene from BmNPV-K1. The PCR primers for amplification of BmNPV-K1 polyhedrin gene were described in Materials and Methods. The amplified 330 bp polyhedrin gene was analyzed by 1% agarose gel electrophoresis (A). Lane 1, *Eco*RI/*Hind*III-digested λ DNA as a molecular size marker; lanes 2 and 3, the amplified 330 bp polyhedrin gene (arrow). (B) The amino acid sequences of the amplified 330 bp polyhedrin gene from BmNPV-K1. The nuclear localization site (KRKK) was indicated in boldface.

to dish. The Lipofectin-DNA complexes were added to the medium covering the cells. After incubating at 25°C for 5 hr, 1.5 ml TC100 medium containing antibiotics and 10% FBS was added to dish and the incubation at 25°C continued. At 5 days postinfection (p.i.), the culture supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4°C. Recombinant BmNPV was plaque purified in Bm5 cells (O'Reilly *et al.*, 1992).

Microscopy

Microscopy of Bm5 cells infected with recombinant BmNPV was performed using a light and fluorescent microscope (Hund H 500).

SDS-polyacrylamide gel electrophoresis (PAGE)

The purified polyhedra and cells infected with recombinant BmNPV were washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and mixed with a protein sample buffer. Total protein samples were performed on a 10% polyacrylamide separting gel with a 3% stacking gel containing SDS as described by Laemmli (1970).

Results

Construction of recombinant BmNPV producing the fluorescent polyhedra

To identify partial polyhedrin gene in BmNPV-K1, we have employed PCR based on the coding region of polyhedrin gene of BmNPV-K1 (Fig. 1). The molecular size of the products in the BmNPV-K1 was identical to that expected and the amplified products were cloned. The nucleotide sequence of PCR products was analyzed and its amino acid was deduced. As the result of the amplified nucleotide sequence in Fig. 1, the 330 bp partial polyhedrin gene has 110 amino acids containing nuclear localization site (KRKK) and its sequence was the minimal

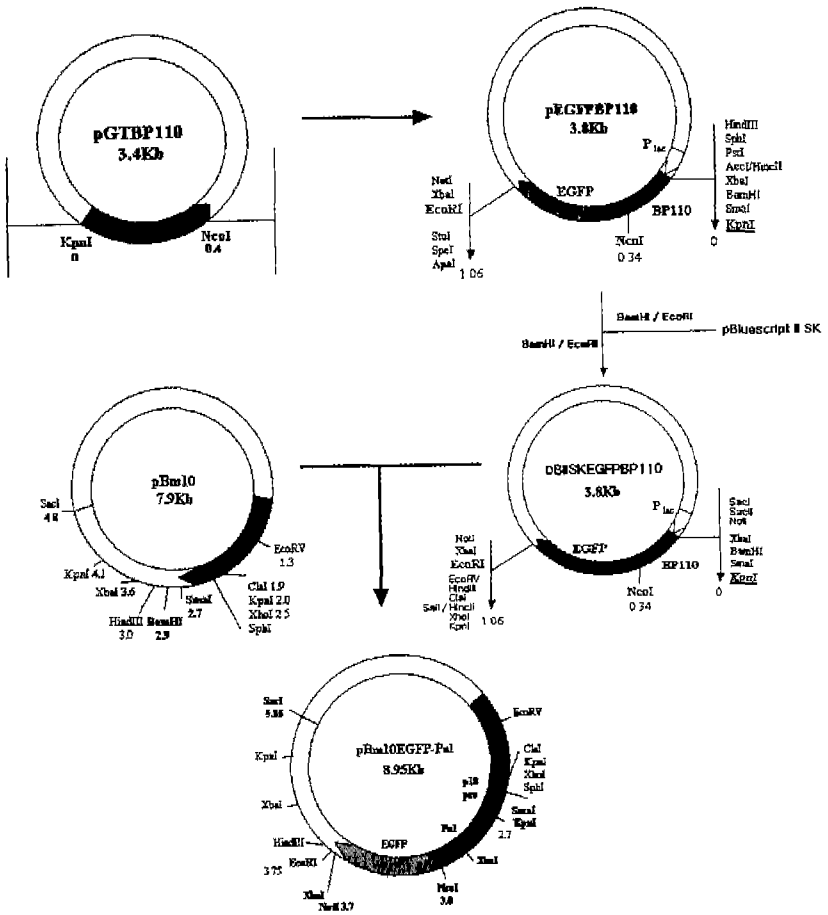


Fig. 2. Schematic diagram of the transfer vector construction for generation of recombinant BmNPV producing the green fluorescent polyhedra. Detailed procedures were described in Materials and Methods. The transfer vector pBm10EGFP-pol was constructed by insertion of partial polyhedrin gene (Pol) and *gfp* gene (EGFP) under the control of BmNPV p10 promoter (p10 pro) into pBm10 (Kang *et al.*, 1997).

polyhedrin gene for forming polyhedra.

The strategy for construction of the transfer vector to generate recombinant BmNPV producing the green fluorescent polyhedra was described in Fig. 2. The *gfp* gene was translationally fused in the back of partial polyhedrin gene of pBIISKEGFPBP110 to produce pBm10EGFP-pol transfer vector. In the pBm10EGFP-pol, the fusion gene was introduced under the control of BmNPV-K1 p10 promoter. The pBm10EGFP-pol transfer vector was cotransfected with the genomic DNA of vBm10β-gal, recombinant BmNPV expressing β-galactosidase under the control of p10 promoter, into Bm5 cells. The recombinant virus was plaque purified in Bm5 cells and named as vBm10pol-GFP.

To determine the formation of recombinant polyhedra, cells infected with recombinant BmNPV vBm10pol-GFP were observed by light and fluorescent microscope (Fig. 3). As expected, cells infected with vBm10pol-GFP produced the green fluorescent polyhedra. Furthermore, the morphology of the purified recombinant polyhedra was similar to that of wild-type BmNPV-K1 (Fig. 4).

To verify whether the recombinant polyhedra were combined with GFP, we examined SDS-PAGE analysis (Fig. 5). As shown in Fig. 5, the purified wild-type Bm NPV-K1 polyhedra showed a molecular weight of polyhedrin typical. The fusion protein expressed by the fusion gene was present as a band of about 41 kDa in cells infected with recombinant virus vBm10pol-GFP and the purified recombinant polyhedra of vBm10pol-GFP, which was absent in the wild-type BmNPV-K1. However, the cells infected with recombinant virus vBm10pol-GFP and the purified recombinant polyhedra of vBm10pol-GFP also showed a 32 kDa native polyhedrin band as wild-type BmNPV-K1.

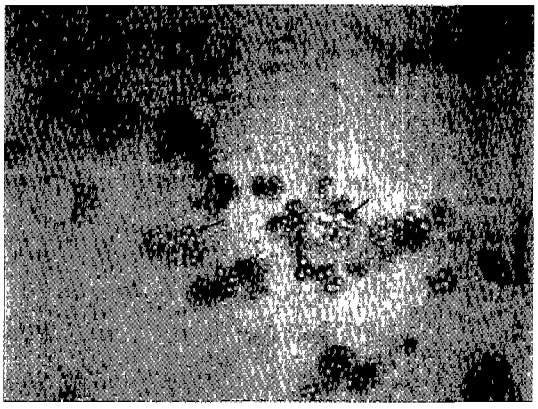


Fig. 3. Microscopy of Bm5 cells infected with recombinant BmNPV vBm10pol-GFP. Cells were observed by the fluorescent microscope (× 200) at 5 days p.i. Arrows indicate recombinant polyhedra with green fluorescence.

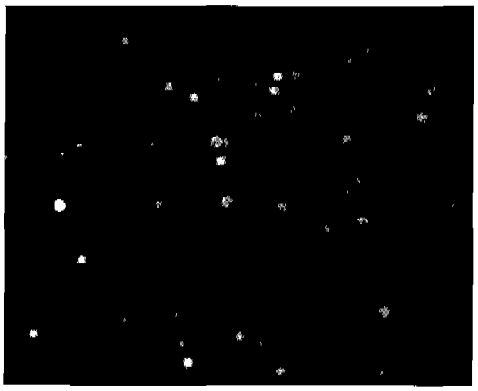


Fig. 4. Microscopy of the purified fluorescent polyhedra of recombinant BmNPV vBm10pol-GFP. The purified polyhedra were observed by the fluorescent microscope (× 200).

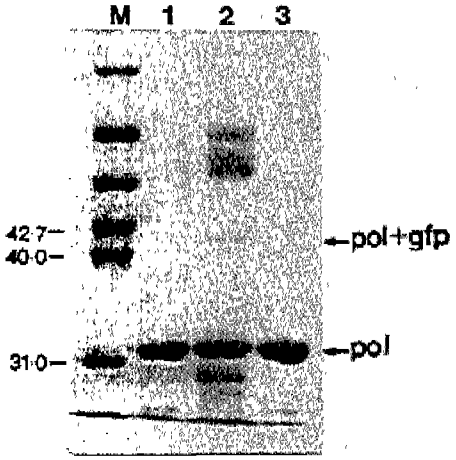


Fig. 5. SDS-PAGE analysis of the fusion protein expression of recombinant BmNPV vBm10pol-GFP in Bm5 cells. Lane 1, the purified polyhedra from wild-type BmNPV; lane 2, cells infected with recombinant BmNPV vBm10pol-GFP at 5 days p.i.; lane 3, the purified polyhedra from vBm10pol-GFP. Polyhedrin (pol) and fusion protein (pol + GFP) bands are indicated on the right of panel. Molecular weight standards were used as a size marker (lane M).

Construction of BmNPV vector producing the polyhedra with foreign gene product

The schematic diagram of BmNPV transfer vector used to generate recombinant virus producing the polyhedra with foreign gene product was described in Fig. 6 and 7. The partial polyhedrin gene, factor Xa, and multiple cloning sites were serially fused and inserted into pinpointXa-2 vector to produce pXa2-pol110 (Fig. 6). The transfer vector pXa2-pol110 has a partial polyhedrin gene for the production of the recombinant polyhedra with foreign gene product, factor Xa for easy purification of foreign product and multiple cloning sites for insertion of foreign gene. To test the function of this transfer vector, the partial poly-

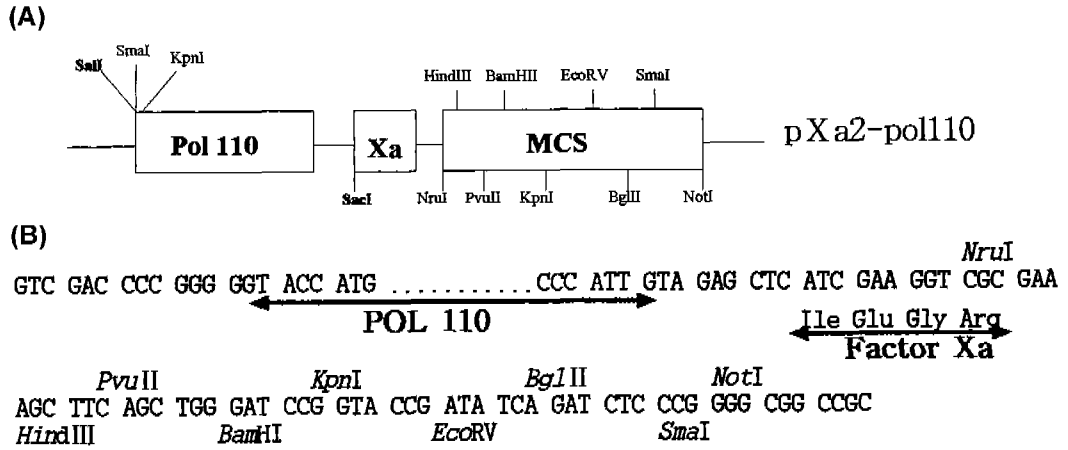


Fig. 6. Schematic diagram (A) and nucleotide sequences (B) of the vector pXa2-pol110. The pXa2-pol110 contained the partial polyhedrin gene (Pol 110), factor Xa (Xa) and multiple cloning sites (MCS).

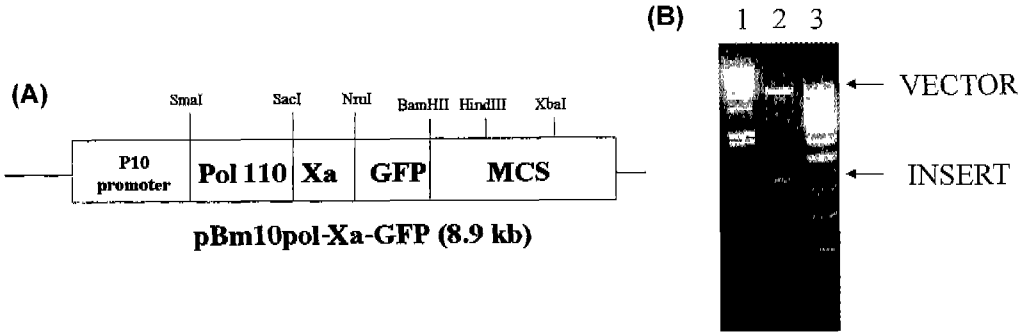


Fig. 7. Schematic diagram (A) and restriction enzyme digestion pattern (B) of the transfer vector pBm10pol-Xa-GFP. The pBm10pol-Xa-GFP was constructed by insertion of partial polyhedrin gene (Pol 110), factor Xa (Xa), and *gfp* gene (GFP) in the multiple cloning sites (MCS) under the control of BmNPV p10 promoter of pBm10 (Kang *et al.*, 1997). The pBm10pol-Xa-GFP was digested with *Sma*I and *Bam*HI restriction enzymes. Lane 1, *Hind*III-digested λ DNA as a molecular size marker; lane 2, pBm10pol-Xa-GFP digested with *Sma*I and *Bam*HI. Arrows indicate the vector and insert in pBm10pol-Xa-GFP.

hedrin gene, factor Xa, and multiple cloning sites were eluted from pXa2-pol110 and introduced under the control of BmNPV p10 promoter of pBm10 vector to produce pBm10pol-Xa. Finally, the *gfp* gene was inserted into the multiple cloning sites of pBm10pol-Xa to yield transfer vector pBm10pol-Xa-GFP (Fig. 7). The exact insertion of *gfp* gene into pBm10pol-Xa-GFP was confirmed by the digestion of the *Sma*I and *Bam*HI.

The pBm10pol-Xa-GFP transfer vector was cotransfected with the genomic DNA of vBm10β-gal, recombinant BmNPV expressing β-galactosidase under the con- trol of p10 promoter, into Bm5 cells. The recombinant virus was plaque purified in Bm5 cells and named as vBm10pol-Xa-GFP. To determine the formation of recom- binant polyhedra, cells infected with recombinant Bm NPV vBm10pol-Xa-GFP were observed by light and fluorescent microscope (Fig. 3). As expected, cells infected with vBm10pol-Xa-GFP produced the green fluorescent polyhedra.

Discussion

We have developed a novel recombinant baculovirus BmNPV producing the green fluorescent polyhedra by fusion of partial polyhedrin gene and *gfp* gene under the control of BmNPV p10 promoter. Furthermore, we have attempted to construct a novel BmNPV p10-based transfer vector producing recombinant polyhedra with foreign gene product.

In the recombinant BmNPV vBm10pol-GFP, the 330 bp partial polyhedrin gene composed of 110 amino acids including nuclear localization site (KRKK) is the minimal polyhedrin gene for forming polyhedra (Jarvis *et al.*, 1991). The strategy for construction of the recombinant BmNPV producing the green fluorescent polyhedra was to fuse *gfp* gene translationally in the back of partial polyhedrin gene and was to introduce under the control of BmNPV-K1 p10 promoter (Kang *et al.*, 1997). In this

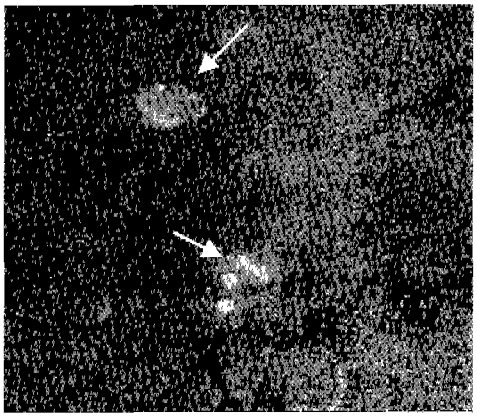


Fig. 8. Microscopy of Bm5 cells infected with recombinant BmNPV vBm10pol-Xa-GFP. Cells were observed by the fluorescent microscope (× 400) at 5 days p.i. Arrows indicate recombinant polyhedra with green fluorescence.

case, cells infected with vBm10pol-GFP produced the green fluorescent polyhedra. Furthermore, the morphology and the size of the purified recombinant polyhedra of vBm10pol-GFP were similar to those of wild-type Bm NPV. However, SDS-PAGE analysis showed that the fluorescent polyhedra had two proteins, fusion protein and only native polyhedrin. In this result, fusion protein and native polyhedrin were respectively produced under the control of p10 and polyhedrin promoters. Although the expression level of fusion protein in vBm10pol-GFP was lower than expected, the polyhedra of vBm10pol-GFP were apparently the recombinant polyhedra with green fluorescence. It was suggested that the native polyhedrin and the partial polyhedrin in fusion protein can provide an important role in the formation of recombinant polyhedra with fusion protein. In this study, stable production of the recombinant polyhedra is possible because native polyhedrin and fusion protein from a recombinant virus can be expressed in the same cell. These results were consistent with the previous reports of AcNPV in that the fluorescent polyhedra are formed coassembly both by the fusion protein with GFP and native polyhedrin expressed in the infected cell (Je *et al.*, 2000, 2001).

To construct the BmNPV transfer vector for recombinant virus producing the polyhedra with foreign gene product, the transfer vector pBm10pol-Xa was constructed by combining partial polyhedrin gene, factor Xa with multiple cloning sites under the control of BmNPV p10 promoter. In the pBm10pol-Xa, partial polyhedrin gene, factor Xa, and multiple cloning sites are for the production of the recombinant polyhedra with foreign gene product, for easy purification of foreign product and for insertion of foreign gene, respectively. For the functional assay of this transfer vector, we formed the fluorescent polyhedra by inserting *gfp* gene into the multiple cloning sites of pBm10pol-Xa. Finally, the constructed vector pBm10pol-Xa-GFP produced the recombinant virus named as vBm10pol-Xa-GFP in Bm5 cells, and cells infected with vBm10pol-Xa-GFP were the green fluorescent polyhedra.

In conclusion, we found that it is possible to construct the recombinant BmNPV producing the polyhedra with foreign gene product. This system subsequently can be applied for the production of recombinant polyhedra with foreign gene product in *B. mori*-derived cells and silkworm larvae.

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